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ARTICLE

A novel mutation of the *RPGR* gene in a Chinese X-linked retinitis pigmentosa family and possible involvement of X-chromosome inactivation

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Abstract

Objectives The objective of this study is to investigate the molecular mechanisms and genotype–phenotype correlations of a Chinese family with X-linked retinitis pigmentosa (XLRP).

Methods A four-generation family with a total of 41 individuals including 7 affected males was recruited. All subjects in this pedigree underwent a complete ophthalmic examination. Targeted capture and next-generation sequencing were performed on the proband using a multigene panel containing 57 known causative genes of retinitis pigmentosa (RP), including *RP1*, *RP2*, *RPGR*, *RHO*, *PRPH2*, *CRB1* among others. All variants were verified in the remaining family members by polymerase chain reaction amplification and Sanger sequencing. Blood DNA was used for X-chromosome inactivation analysis in female carriers.

Results All the affected individuals were diagnosed with RP. The affected males showed symptoms from the first decade, while the female carriers had onset in the second decade or later. A frameshift mutation c.345_348delTGAA in the *RPGR* gene was identified in all affected males and female carriers. By XCI analysis, we found that there was little correlation between their phenotype and the methylation status of their X chromosomes.

Conclusions A novel mutation c.345_348delTGAA of the *RPGR* gene was identified, expanding the spectrum of *RPGR* mutations causing XLRP. In this pedigree, the phenotype extended to female carriers, in whom RP was milder and its onset delayed compared to hemizygous males. Although lack of strong correlation between X-inactivation and the severity of the disease, the milder, variable effects in female carriers still could reflect X-inactivation patterns in the retina of each individual.

Introduction

Retinitis pigmentosa (RP; OMIM 268000) is a group of inherited retinal dystrophies characterized by progressive loss of photoreceptors, classified as non-syndromic and syndromic depending on whether the disease is confined to the eye or not. The prevalence of non-syndromic RP is ~1/4000 [1, 2], which encompass about 65% of all RP cases. Clinically, it can be divided into three stages. Night

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blindness (nyctalopia) is usually the earliest symptom, reflecting rod photoreceptor cells being the first affected cell type in the retina. The visual acuity of RP patients tends to be unaffected until the middle stage of the disease, which often also shows a loss of peripheral vision even in day light. With further progression the visual field constricts further, evolving to a ring shape scotoma and then tunnel vision, eventually leading to legal blindness by about the fourth decade. In typical cases, the fundus shows the triad of RP including bone spicule-shaped pigment deposits, waxy pallor of the optic disc, and attenuation of the retinal vessels. Correspondingly, the a and b waves of the electroretinogram (ERG) are diminished or even undetectable. However, the age of onset, disease progression, and severity of symptoms can vary markedly among different patients, even in the same pedigree.

The inheritance patterns of RP include autosomal dominant (ADRP, 15–25%), autosomal recessive (ARRP, 5–20%) [3], X-linked (X-linked retinitis pigmentosa (XLRP), 5–15%), and digenic forms (rare) [2, 4, 5]. Among those, the autosomal dominant form is usually mildest, with some patients presenting with delayed onset in their early 50 s. XLRP tends to be the most severe type with an early onset and rapid progression [6]. Many XLRP cases are accompanied with myopia [7, 8]. Digenic inheritance is rare, and it usually inherited in a pseudo-dominant form. Meanwhile, 40–50% of RP cases are sporadic with an unknown inheritance pattern. In addition, many families with XLRP show an X-linked mode of inheritance in which affected males usually manifest early onset severe disease with rapid progression, while female carriers are asymptomatic or only minimally affected [9, 10].

The RP GTPase regulator gene (*RPGR*, OMIM 312610), located at chromosome Xp11.4, was the first causative gene for XLRP identified in 1996 [11]. The other two known XLRP causing genes are *RP2* (10%–20%) and *OFD1* (rare), respectively [12, 13]. At this time, more than 300 variants in *RPGR* have been identified [14]. The RPGR protein is concentrated in photoreceptor connecting cilia and interacts with another ciliary protein, RPGRIP1 [15, 16]. Clinical observation of patients, biochemical studies, and animal models suggests that the RPGR protein plays an important role in the transport of phototransduction components and other outer segment proteins across the connecting cilium [14].

Female carriers of *RPGR* mutations are usually asymptomatic or show mild phenotypes due to the X-linked inheritance of the disease. However, carriers can manifest a wide spectrum of clinical symptoms ranging from asymptomatic to varying extents of RP abnormalities [17, 18]. Freidrich et al. [19] described a XLRP family caused by mutant *RP2*, in which the clinical features of female carriers were highly variable, termed semidominant XLRP. It has been reported that the variable phenotype of female carriers reported in other studies with the *RP2* or *RP3* disease-causing genes may be due to variable X-chromosome inactivation (XCI) or other relative factors [20, 21].

In this study, a Chinese pedigree segregating XLRP was described and a deletion, c.345_348delTGAA, was identified in exon 5 of *RPGR* via targeted capture and next-generation sequencing (NGS) [22, 23]. Since skewed inactivation of the X-chromosome, with overexpression of the mutant gene in symptomatic females, could theoretically be the cause of retinal phenotype, XCI analysis by assaying methylation status of a polymorphic repeat at the androgen receptor (AR) was carried out in symptomatic and asymptomatic female carriers.

Materials and methods

Patients and clinical examination

The study was approved by the Medical Ethics Committee of Shenzhen Eye Hospital in Shenzhen, Guangdong, China. Informed consent was obtained from all participants according to the principles of Declaration of Helsinki. No consanguineous marriage was noticed in the family.

Routine ophthalmic examinations, including visual acuity measurements with linear Snellen, slit-lamp examinations, and fundus photography, were conducted in each individual. The proband was examined by electroretinography (ERG) using Roland Consult RETIport ERG system, fluorescence fundus angiography (FFA) using Heidelberg Retina Tomography, visual field using Humphrey field Analyzer, and optical coherence tomography (OCT) using Carl Zeiss Cirrus 4000 HD OCT.

Criteria for the diagnosis of retinitis pigmentosa

Diagnosis of RP was made based on functional signs including decreased night vision or night blindness and findings in the fundus including waxy optic disc pallor, narrowing arterioles, and pigment deposits. Visual field and electroretinography (ERG) were used to assess the stage of RP. OCT was used to analyze the retinal pigment epithelium and the retinal nerve fiber layer thickness in the retina.

Target capture sequencing

2 ml peripheral venous blood was collected from all study subjects, which was used to extract Genomic DNA using a QIAmp Blood DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer protocols. The integrity of the extracted DNA was verified by 1% agarose gel electrophoresis. Since there were no ocular or other disorders noticed other than RP in this family, 57 candidate genes for non-syndromic inherited RP including the *RPGR* gene were

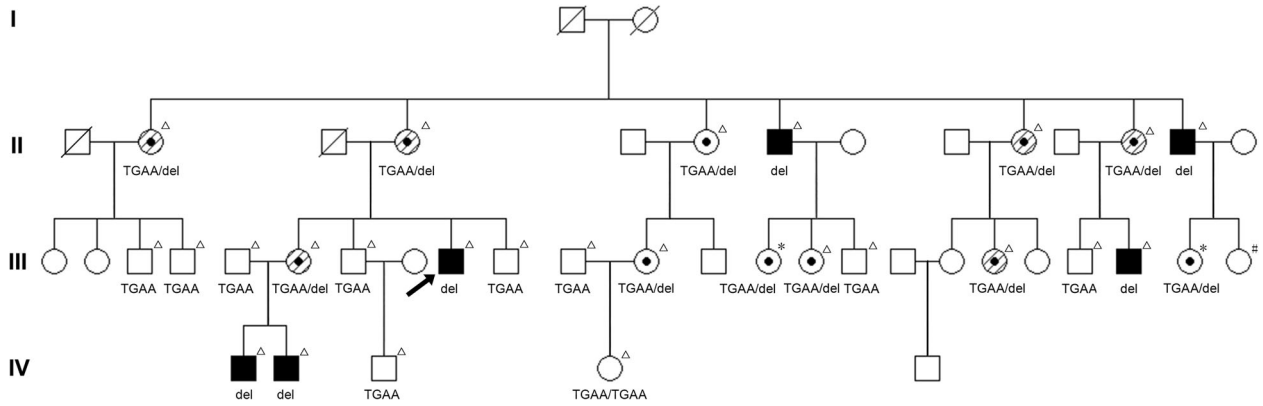

Fig. 1 A Chinese pedigree with X-linked retinitis pigmentosa. Square symbols denote males; the circles denote females. The filled shapes indicate the affected individuals with RP, a dot in the middle of the circle indicates an asymptomatic carrier in the family and a dot in the middle of the circle with slant line inside indicates a symptomatic carrier. The arrow signifies the proband. The symbol △ denotes the individual who was both clinically examined and genotyped. The symbol * denotes the individual who was only genotyped. The symbol # denotes the individual who was not clinically examined and genotyped because of her very young age (2 years old).

included in the designed sequencing panel (Supplementary File 1) using custom-designed NimbleGen SeqCap probe hybridization (Roche NimbleGen, Inc., Madison, WI, USA), with plans to carry out WES if no changes were found in the panel genes. Target capture sequencing of all coding exons and 20 bp of herein flanking intronic regions of these genes were performed on the proband to detect the possible disease-causing mutations. DNA samples with equal molar ratios were put into each flow cell along the displayed lanes. Polymerase chain reaction (PCR) was observed with HiSeq2000 (Illumina, Inc., San Diego, CA, USA) using the sequencing-by-synthesis method.

Alignment and variant calling

The sequencing reads were aligned to the human reference genome (GRCh37/hg19) with SOAPaligner and BWA separately. SOAPsnp v1.05 was then applied to the alignment resulted from SOAPaligner, and GATK v3.30 was applied to alignment produced by BWA to call variants. Identified variants were filtered against the dbSNP 129, 1000 genome project, and Human Gene Mutation Database (HGMD).

Verification of variants

Sanger sequencing was conducted to confirm the indicated mutations to determine whether any of the remaining variants co-segregated with the disease phenotype in this family. PCR primers of the likely pathogenic variants including the one in exon 5 of the *RPGR* gene (Forward: 5′-GGACTCTATAGTCTATTGACGTT-3′; Reverse: 5′-AGGAATGTGTCCCAGACTGAA-3′) were designed by the Primer Premier 5 software. PCR amplification was performed. Purified PCR products were sequenced using an ABI 377XL automated DNA sequencer (Applied Biosystems, Foster City, CA). All variants were interpreted and classified based on the nomenclature recommended by the Human Genomic Variation Society.

X-chromosome inactivation (XCI) analysis

To evaluate the inactivation pattern of the X-chromosome, XCI analysis was performed on six symptomatic female carriers and five asymptomatic female carriers. DNA samples were obtained from peripheral venous blood as above. The X-inactivation assay of *AR* and *RP2* locus using *Hpa*II-sensitive methylation was performed according to previous reports [24, 25]. For each sample, 500 ng of DNA was digested with *Hpa*II restriction endonuclease (New England Biolabs, Ipswich, MA). Digested products together with non-digested DNA were used as templates for amplification of the *AR* and *RP2* polymorphic repeat using fluorescence labeled primers [24–26]. PCR fragments were run in an ABI 3500xL Genetic Analyzer (Applied Biosymtems, Foster City, CA). *AR* and *RP2* alleles were sized and quantified using Genemapper software (Applied Biosymtems, Foster City, CA). To correct preferential allele amplification, the allele ratio in *Hpa*II digested DNA was normalized using the ratio of non-digested DNA. As described in the literatures [27, 28], XCI ratios (active mutant RPGR to active wild-type RPGR) of ≤80:20 were considered “random” pattern, ratios greater than 80:20 were considered “highly skewed” pattern.

Results and discussion

Clinical findings

A four-generation family with a total of 41 members originating from the remote rural areas in Southwest

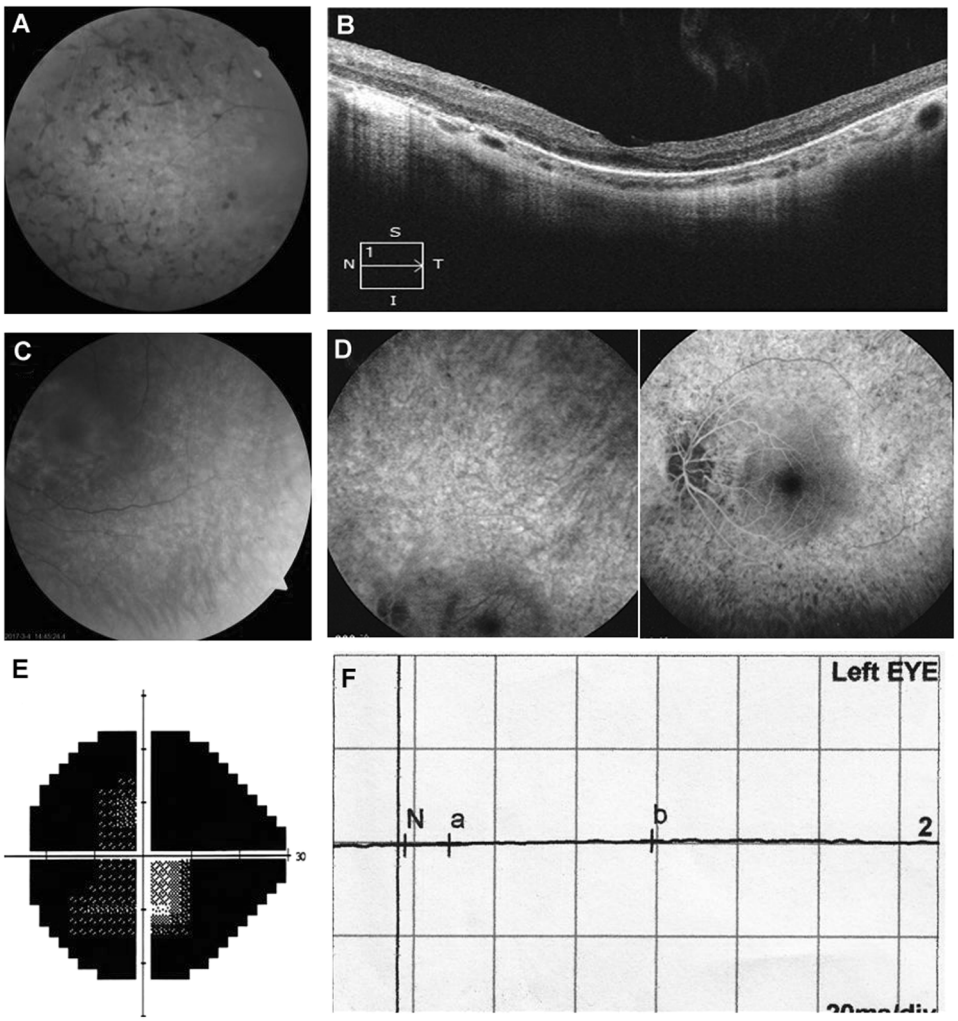

Fig. 2 Representative photographs of patients of this family. Fundus photographs showed pigmentary changes with bone spicule-like pigmentation (**a**, patient II7) and peripapillary chorioretinal atrophy (**c**, patient III9). **b** OCT of patient III9 revealed marked atrophy of the retina pigment epithelium and loss of photoreceptors. Cystic cavities were visible in the inner and outer nuclear layers and epiretinal membrane. **d** Fundus fluorescein angiography (FFA) demonstrated narrow retinal vessels and chorioretinal atrophy (patient III9). **e** Visual field testing presented only small islands of vision in the far peripheral field was remained (patient III9). **f** ERG responses were almost undetectable, with reduction in amplitude and disappearance of the b-wave.

China was recruited in this study (Fig. 1). Twenty-four individuals including 5 affected males and 6 affected heterozygous females were evaluated by clinical examinations. Patient II13 was unavailable for clinical examination because of far distance, but a detailed medical history was collected during our visit which confirmed that he had a typical clinical phenotype of RP (data not shown).

The proband, a 24-year-old male, had night blindness and progressing visual field and visual acuity impairment since childhood. On his most recent visit, his best corrected visual acuity was 0.3 (−5.25DS − 1.50DC × 150) in the left eye and no light perception (NLP) in the right eye, which suffered from a severe trauma years ago. For the left eye, fundus examination showed waxy pale disc, bone spicule-shaped pigment deposits in the mid periphery and retinal arteriolar attenuation. ERG recordings demonstrated no detectable cone or rod responses. OCT showed retinal atrophy and fundus fluorescein angiography (FFA) exhibited narrow retinal vessels and chorioretinal atrophy in the periphery. Visual field testing showed that only small islands of vision close to the fixation point remained.

All the affected males in the family presented with typical features of XLRP (Fig. 2). They presented with night blindness and poor visual acuity since the first decade of life, and showed typical fundus features for RP, such as pigmentary changes with bone spicule-like pigmentation, attenuation of the retinal vessels, and chorioretinal atrophy.

There were 11 female carriers included in this study. Two of them were not able to be examined clinically, but a detailed medical history was collected during our visit and at intervals afterward. As they showed no signs or symptoms by the ages of 13 and 19, they were assumed to be unaffected. Most female carriers in the family showed delayed onset of signs and symptoms compared with the affected males. The phenotype of the female carriers varied from totally normal eyes through mild retinal changes to complete loss of vision (Fig. 3). Six female carriers had abnormalities of the retinal pigment epithelium, such as pigment clumping and pigment epithelial atrophy. Bone

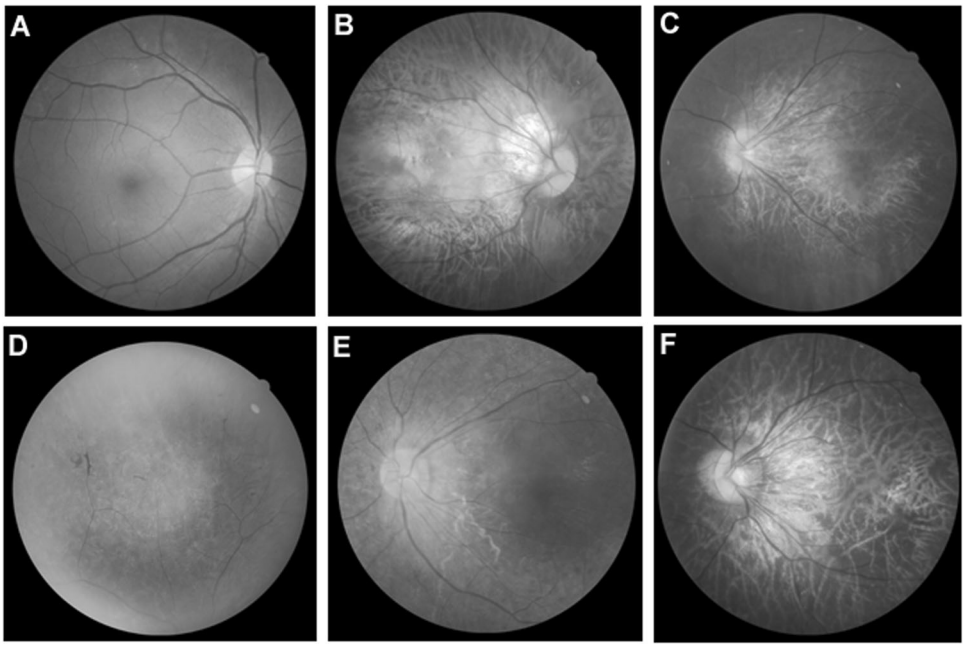

Fig. 3 Representative fundus photographs of the female carriers of this family. Female carriers in this family showed various manifestations of retinal degeneration, from no significant change (**a**) to retinal atrophies (**b**, **c**, **f**) with relatively mild typical RP phenotype (**d**, **e**). **a** II6; **b** II10; **c** II12; **d**, **e** III6; **f** III19.

Table 1 Clinical finds in female carriers.

Individual	Age (years)	Mode of onset	Visual acuity		Fundus
			OD	OS	
II2	61	Night blindness, myopia	NLP	CF/30 cm	RP, MF
II4	53	Night blindness, myopia	CF/20 cm	CF/30 cm	RP, MF
II6	45	Myopia	0.9	0.7	Normal
II10	43	Myopia	CF/30 cm	CF/45 cm	MF, macular hemorrhage
II12	40	Night blindness, Myopia	0.1	0.1	RP, MF
III6	38	Night blindness, Myopia	0.1	0.2	RP, MF
III15	11	N/A	0.7	1.5	Normal
III19	21	Myopia	0.1	0.06	RP, MF

MF myopic fundus for short.

Table 2 Clinical finds in affected males.

Individual	Age (years)	Mode of onset	Visual acuity		Fundus
			OD	OS	
II7	48	Night blindness, myopia	CF/30 cm	CF/20 cm	RP, MF
III9	24	Night blindness, myopia	NLP[a]	0.3	RP, MF
III22	7	Night blindness	0.5	0.6	RP
IV1	9	Night blindness	0.3	0.4	RP
IV2	7	Night blindness	0.7	0.6	RP

[a]This patient had a trauma history of the right eye.

spicule-like pigmentation was found in only one female. Moderate-to-high myopia was recorded in seven female carriers with the mutation of the *RPGR* gene (Table 1). Individual III12 was examined clinically and showed no signs or symptoms and was thus not included in Table 1. Fundus examination also showed changes consistent with myopia including macular degeneration, peripapillary, and chorioretinal atrophies.

The clinical findings of this pedigree are summarized in Tables 1 and 2.

Genetic analysis

Targeted capture and NGS were performed on the proband to analyze a total of 57 candidate genes involved in non-syndromic RP, including *RP1*, *RP2*, *RPGR*, *RHO*, *PRPH2*,

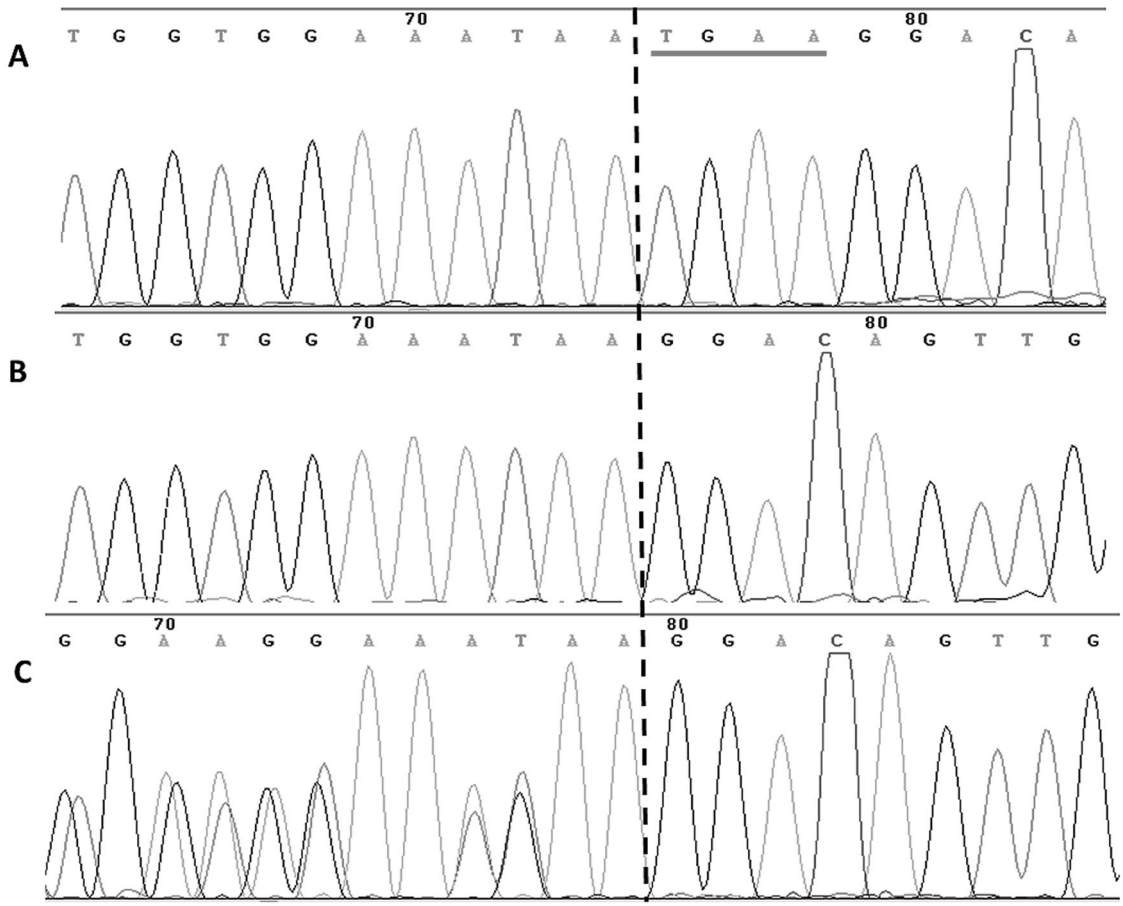

Fig. 4 Sequence results of the *RPGR* gene (exon 5) in this pedigree. **a** a normal individual (III7). **b** a male patient (III9) harbored a hemizygous mutation c.345_348delTGAA of *RPGR* gene. **c** a female carrier (II10) harbored a heterozygous mutation c.345_348delTGAA of *RPGR* gene (red line indicates the mutation site). (color figure online).

and *CRB* et al. (Supplementary File 1). An average depth of ×223.65 and coverage of 99.71% were obtained from the sample of patient III9 with 97.54% of the target region covered by at least 30X, indicating that sufficient depth and coverage were obtained for variant calling. A likely pathogenic mutation, which is a hemizygous one in exon 5 of *RPGR* (c.345_348delTGAA), was identified after filtered by database 1000 genome, dbSNP, and HGMD (frequency < 0.01). This mutation was verified by Sanger sequencing and co-segregated with the retinal phenotype in all affected males (Figs. 1 and 4). Some female carriers with a heterozygote c.345_348delTGAA variant of *RPGR* presented with clinical symptoms, although they were milder and with later onset than their male relatives.

XCI analysis

Skewed inactivation of the X-chromosome, with overexpression of the mutant gene in affected females, could theoretically be the cause of retinal phenotype. In order to verify whether skewed X-inactivation may be involved in the pathogenesis of disease in female carriers in this family, we conducted XCI analysis in the peripheral blood of the 11 female carriers. XCI ratios (active mutant RPGR to active wild-type RPGR) ranged from 0.44 (30.6:69.4) to 4.44 (81.6:18.4), with median 1.19 (54.4:45.6). Eight out of 11 (72.7%) had XCI > 50:50 and only one affected individual showed an inactivation level of >80:20 which is highly skewed. We compared the XCI ratios with the clinical severity of RP phenotypes of each female carrier including the visual acuity and presence and severity of retinal (macular) degeneration. For each affected individual, the severity of visual acuity loss and night blindness is consistence with that of his or her degenerative retinopathy. However, although it lacks strong correlation between the clinical phenotypes and the degree of degenerative retinopathy, there was little correlation between the phenotypes, including the visual acuity and presence and severity of retinal (macular) degeneration, and the methylation status of the X chromosomes in the patients overall. This might due

to the fact that the sample size was relatively small and X-inactivation levels in the blood was not that representative for that in the retina. In addition, other factors might contribute to variation in the phenotypes from asymptomatic to varying extent of RP abnormality.

Discussion

Genetic analysis suggests that RP in this Chinese family is inherited in a semidominant XLRP inheritance pattern. A novel frame-shifted mutation, c.345_348delTGAA in exon 5 of the *RPGR* gene, was identified by targeted-capture NGS in this study. This mutation led to a severe RP phenotype in affected males, with significant variability in the age of onset of night blindness and visual acuity in female carriers.

RPGR was the first gene associated with non-syndromic XLRP. *RPGR* contains 19 exons encoding 1020 amino acids and has at least 12 isoforms [29], most of which are tissue specific, indicating that they might play different roles in the specific tissues. The isoform *RPGR* exons 1–19 and *RPGR* ORF15 are the two major *RPGR* isoforms [30], the first accounts for only 15–20% of XLRP, while the latter accounts for at least 55% of XLRP cases [31]. Even though the detailed structure and function of these isoforms are not fully understood, it is generally accepted that the *RPGR* protein plays an important role in maintaining the function of cilia [30, 32]. In addition to XLRP, X-linked cone dystrophy, cone-rod dystrophy, and atrophic macular degeneration also can result from mutations in *RPGR* [33–35]. Mutations in *RPGR* can cause dysfunction of other organs including syndromic primary ciliary dyskinesia, a multisystem disorder characterized by recurrent respiratory tract infections, sinusitis, bronchiectasis and male subfertility coexisting with RP [36]. In this study, there were no constitutional symptoms, although myopic changes including macular degeneration, peripapillary atrophy, and chorioretinal atrophy were found in female carriers. These observations are consistent with prior descriptions that mild to high myopia were frequently seen in female carriers as well as in male affected individuals [7, 8].

The c.345_348delTGAA mutation of *RPGR* results in a four bp-deletion in exon 5 of *RPGR*, which is predicted to result in a truncated protein product (p.Asn115LysfsX17), affecting both the *RPGR* exons 1–19 and *RPGR* ORF15 isoforms [37, 38]. By comparing the visual fields and 30 Hz ERG amplitudes of age-matched patients with *RPGR* ORF15 mutations with those of patients with *RPGR* exons 1–14 variants, Sharon et al. demonstrated that the patients with ORF15 mutations may retain partial function, and showed the disease severity varied according to the predicted length of the encoded abnormal amino acid sequence [12]. Bader et al. and Fahim et al. also came to a similar conclusion that patients with upstream mutations in exons 1–14 show a more severe phenotype [39, 40]. Thus, the mutation c.345_348delTGAA, involving both isoforms, could explain the severe phenotype observed in this family.

In this study, all the male patients showed night blindness and visual acuity decreasing from their childhood. Poor visual acuity, as well as constricting vision and nearly undetectable ERGs, was present beginning in the second decade, accompanied by obviously bone spicule-like pigmentation on fundus examination. In contrast, some female carriers present with myopia with or without certain degree of RP associated abnormalities, including macular degeneration, peripapillary atrophy, and chorioretinal atrophy. The clinical features of female carriers show a broad spectrum of fundus manifestations, from merely mild myopic retinopathy to typical pigmentary deposits. Moreover, a delayed onset was noted in those females with fundus abnormalities. No abnormalities were seen until the second decade, and classical fundus signs of RP were absent in their first 13–14 years of life. The clinical severity and disease phenotype varied from asymptomatic to high myopia to more classical RP among affected females in this family, even though they had the same pathogenic mutation, as has been noted in some previous studies [41–43].

Most *RPGR* mutations have been considered to be recessive with female carriers clinically unaffected or having a mild phenotype [20, 44, 45]. However, severely affected heterozygous carriers have been reported [17, 20]. In the pedigree reported in this study, the phenotype was milder and delayed in female carriers compared to hemizygous males. This form of X-linked RP could be regarded therefore as partially dominant or semidominant. As reported in the literature, skewing of X-inactivation may be responsible for varying clinical severity in XLRP female carriers. Fahim et al. demonstrate a significant effect of XCI skewing on disease severity in carriers of XLRP [26]. However, we found little correlation between their phenotypes and the methylation status of their X chromosomes in blood. In this study, some female carriers with an XCI ratio < 80:20 still had milder and variable phenotype, suggesting that other factors are contributing, potentially could be related to the following points. First, the sample size is relatively small so that there is no statistical power. Second, genetic and epigenetic analysis was carried out in blood DNA because of the relative inaccessibility of retinal tissue. Although blood had been used as a proxy for other tissues in determining XCI ratios, blood XCI skews with age out of proportion to other tissues [46] and may not accurately reflect retinal XCI ratios. Lastly, since genome sequencing was not performed, other genes

mutations may contribute to the varied phenotype of the female carriers.

Conclusion

A novel frame-shifted mutation, c.345_348delTGAA in exon 5 of the *RPGR* gene, was identified as the cause of XLRP in this study. To the best of our knowledge, this is the first report that this mutation in *RPGR* is responsible for the pathogenesis of XLRP. Although lack of strong correlation between the methylation status of the X chromosomes in the patients and the severity of the disease, varying XCI in the retina might be a reasonable explanation for the milder variable signs of disease in female carriers.

Summary

What was known before

- Female carriers of RPGR mutations are usually asymptomatic or show mild phenotypes due to the X-linked inheritance of the disease.

What this study adds

- A novel frame-shifted mutation, c.345_348delTGAA of the RPGR gene, was identified as the cause of XLRP in this study.
- The phenotype extended to female carriers in this pedigree, in whom RP was milder and its onset delayed compared to hemizygous males.
- The milder, variable effects in female carriers in this XLRP family still could reflect X-inactivation patterns in the retina of each individual.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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